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Severe hemorrhage is associated with increased intracellular accumulation of sodium and water, reflecting membrane depolarization i.e. decreased activity of the sodium pump. Since others have described normal or increased ATP during this depolarization, we hypothesize that Na/K ATPase is inhibited in shock. Since the defect is seen in red blood cells, a circulating factor appears to be involved. We have identified this factor as the 33amino acid C-terminal fragment of Plasminogen Activator Inhibitor type-1 (PAI-1) generated by the cleavage of PAI-1 at its Arg-Met active center by plasminogen activators. Synthetic 33aa fragment causes dose-dependent inhibition of Na/K ATPase in membrane vesicles. (We call the fragment Sodium Pump Inhibiting Peptide, SPIP). We have measured SPIP in the plasma of rats in hemorrhagic shock and have found the apparent concentration to be sufficient to inhibit completely membrane Na/K ATPase (compared to ouabain). We conclude that hemorrhage elevates a form of PAI-1 that is bound and cleaved by plasminogen activators producing SPIP. SPIP, in turn, inhibits membrane Na/K ATPase producing cell depolarization and increased cell sodium and water, contributing to the development of hemorrhagic shock.

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Introduction and Background

Hemorrhage leads to restitution of blood volume through two principal mechanisms. First, hemorrhage leads to a fall in arterial blood pressure and reflex vasoconstriction. The resulting fall in capillary hydrostatic pressure leads to movement of protein-free fluid from the interstitial space to the vascular space (1,15,47,79,86). Second, the splanchnic production of solute, mediated by the multihormonal response to volume loss, leads to a transient increase in extracellular osmolality (12). As a result, water moves from the intracellular space to the extracellular space, increasing interstitial and plasma volume and pressure (36,37,65,66,83). There is an associated change in the access of albumin to the intravascular compartment, so that there is net movement of albumin and fluid to complete the restitution of blood volume (65,66).

If the magnitude of hemorrhage exceeds 25%, the above mechanism fails, and complete restitution of blood volume does not occur (14,36). We have shown that this failure occurs despite the continued production of solute (36), suggesting that some other mechanisms come into play that offset the osmotically driven shift of fluid out of the cells. Evidence from Shires' laboratory suggests that cells depolarize throughout the body (with a decrease of transmembrane potential of about 30%, 75,76). As cells depolarize, sodium and water accumulate. The accumulation of intracellular water and solute counteracts the mechanisms involved in restoration of blood volume. Without complete restitution, cardiovascular compensation fails, so that cardiac output remains depressed, and peripheral resistance remains high (13). The interaction of cell depolarization and the physiologic mechanism for blood volume restitution leads to one possible view of cardiovascular shock: Because of the sequestration of intracellular fluid after a severe cardiovascular insult (i.e., decreased effective blood volume) cardiovascular stabilization cannot occur. This phase of "shock" can be treated by fluid resuscitation. The link to a later phase of so called "irreversible shock", in which fluid resuscitation is not effective and cell injury and death occurs, is not clear at this time.

Depolarization of cells after hemorrhagic shock has been generally attributed to cellular hypoxia resulting from ischemia and to a decrease in the production of ATP (4,7,17,18). However, we have shown that the appearance of cell depolarizing activity in plasma following both a non-lethal hemorrhage (rat and dog) and sepsis (rat) occurs rapidly in hemorrhage and precedes cardiovascular destabilization in sepsis (29). Furthermore, Shires *et al.,* (76) have shown that ATP does not decrease in cells from animals in shock, suggesting that shock does not affect intracellular energy supply. They have also shown that shock will depolarize red blood cells, that lack oxidative metabolism (75,76). Finally, we have shown that hypoxia is not required for depolarization to occur in the target cell (33). This evidence strongly suggest that cell depolarization in shock does not depend upon cellular hypoxia secondary to a decrease in oxygen delivery.

We have shown that plasma derived from shocked rats or dogs depolarizes cells (10,11,29,30,33) and depresses myocardial function (48). Using sizing columns, we found that the activity was associated with a fraction of plasma at 200kDa. This fraction

run on SDS-PAGE gel showed bands at approximately 50 and 80kDa. We deduced that a complex of the 50 and 80kDa pieces (plus other proteins) made up the 200kDa fraction. The amplitude of depolarization induced by the 200kDa fraction of shock plasma titrates and saturates with increasing concentration and yields an apparent affinity of 1 nM (33). Treatment with trypsin or chymotrypsin destroys activity. The 80 kDa peptide has been sequenced by Dr. R. Vandlen at Genentech, Inc. and proved to be rat vitronectin. Both the purified 80 kDa peptide and commercially obtained Vn exhibit only slight depolarizing activity. A literature search (69,70) revealed that Vn strongly associates with and activates PAI-1, a 54 kD acute phase protein. Commercial PAI-1 depolarizes red blood cells in a dose-response manner with an apparent K_M of PAI-1 of 1000 nM. Addition of vitronectin decreases the K_M to 100 nM and the presence of both vitronectin and 0.67 mM Fe^{3+} decreases the K_M to 1 nM. While the PAI-1/Vn/ Fe^{3+} mixture or complex induces depolarization with a physiologically reasonable affinity, the maximum amplitude of depolarization is only about 20-30% of that induced by the original 200kDa fraction from shock plasma. This suggested that another factor was involved and may be related to PAI-1.

PAI-1 binds specifically to tissue plasminogen activator (tPA) or to urokinase (urine-derived plasminogen activator, uPA) (71). Both uPA and tPA in low ratios (0.1 PA:PAI-1 or lower) enhance the depolarizing activity of PAI-1. Only about 10% of the enhanced activity appears after a short (2 min) incubation, but activity increases greatly over 2 hours. Thus, tPA and/or uPA are involved in the generation of depolarizing activity. These results suggested an interaction between PAI-1 and PA's as a critical step in the generation of the principal factor mediating cell depolarization.

PAI-1 is present in three forms: active, latent and substrate (25). The active form binds covalently and inhibits the serine proteases, tPA or uPA (54,59). The latent form does not bind or inhibit PAs because of a conformation change in the reactive-center loop located 30-40aa from the C-terminus, preventing binding (2,58). The substrate form binds tPA or uPA but does not inhibit the plasminogen activators. Instead tPA or uPA cleaves the 54kDa PAI-1 to 50 and 4kDa fragments (3,25,61). Substrate PAI-1 appears to have a conformational change in the reactive-center loop (6), but this change allows binding to PAs (40) and cleavage to 50 and 4kDa forms. This 4kDa product is 33 amino acids derived from the C-terminus of PAI-1 as a result of cleavage at an Arg-Met bond by plasminogen activators (3,25,61). The amino acid sequence is as follows:

NH_2-**Met-Ala-Pro-Thr-Glu-Met-Val-Leu-Asp-Arg-Ser-Phe-Leu-Phe-Val-Val-Arg-His-**
    1   2   3   4   5   6   7   8   9   10 11 12 13 14 15 16 17 18
Asn-Pro-Thr-Glu-Thr-Ile-Leu-Phe-Met-Gly-Gln-Leu-Met-Glu-Pro-COOH
 19 20 21 22 23 24 25 26 27 28 29 30 31 32 33

The discovery of the 33aa fragment intrigued us as separation of partially purified shock-plasma on an affinity column of modified urokinase (that binds PAI-1) showed more activity in the flow-through than in the material placed on the column or in the material

retained by the column. In other experiments, using continuous elution SDS electrophoresis, most of the activity was in the low molecular weight fraction (<10kDa) thereby pointing us to the 33aa cleavage product.

Plasminogen activators and PAI-1 are present in both plasma and endothelial cells. At this time, we do not know if SPIP is produced and secreted by cells and/or if SPIP is produced in plasma from the interaction of PAI-1 and tPA or uPA. Therefore, it is necessary to measure SPIP and total PAI-1 by ELISA, as PAI activity is an index only of active PAI-1.

SPIP and PAI-1 antigen are currently measured with a direct ELISA using a polyclonal rabbit anti-rat SPIP IgG directed against a fragment of SPIP (10aa - RSFLFVVRHN - near the N-terminus, developed by Anaspec, Inc.). This 1° antibody cross-reacts with both PAI-1 and SPIP. Therefore, the ELISA will measure both PAI-1 and SPIP. To separate SPIP for PAI-1, plasma is run over an anhydro-urokinase column (87) that binds PAI-1 but not SPIP. ELISA for SPIP is run on the flow through. We use hrPAI-1 generously donated by Dr. Harry L. Walton, Du Pont Merck Pharmaceutical Company, to standardize the total PAI-1 assay, and the synthetic SPIP produced by the University of Maryland School of Medicine Biopolymer Laboratory to standardize the SPIP assay.

Because this antibody was produced with low titers, additional antibodies specifically directed against the amino acids 10-19 and 24-33 of SPIP are currently under development by Hazelton, Inc. The antigens were made synthetically at the University of Maryland Biopolymer Laboratory by coupling multiple copies of the 10-19 or 24-33 fragments to poly-lysine molecules. These antibodies will be used to develop a sandwich ELISA for measurement of SPIP and PAI-1 in plasma and tissue homogenates.

We have recently found that SPIP (synthesized by Dr. N. Ambulos of the Biopolymer Laboratory at the University of Maryland School of Medical) inhibits the Na^+/K^+ ATPase directly and in membrane vesicles in a dose dependent manner with activity in nM concentration as great as that of ouabain in μM concentrations. Because of this finding, we strongly believe that the SPIP is the humoral agent in plasma that causes depolarization of cells by directly or indirectly inhibiting the Na^+/K^+ ATPase pump and thereby leads to the early pathophysiological features of shock described by Shires (75,76).

It has been reported that plasma PAI activity is elevated after septic shock in man (64,68) and pig (77), anaphylactic shock in rabbits (74) and cardiac by-pass surgery in man (16). We have now shown that both PAI activity in plasma and PAI-1 mRNA in liver are elevated following both hemorrhage and sepsis in rat liver (89). We have also shown that hemorrhage and sepsis stimulates transcription of PAI-1 in endothelial and mesothelial cells of rat liver (88). These findings raise questions about what humoral factors mediate the rise in PAI-1 transcription and secretion after hemorrhage. TNF, IL- 1, IL-6, angiotensin II, Transforming Growth Factor-β and glucocorticoids, all have been shown to stimulate synthesis of PAI 1 and PAI-1 mRNA in endothelial and mesothelial cells,

and hepatocytes (26,35,39,41,42,45,46,55-57,60,67,72,78,90). Furthermore, many of these substances are elevated after hemorrhage and shock (20,21,63,80,82). We have set out to determine which humoral agents mediate the rise in PAI-1 mRNA and secretion after hemorrhage. We have recently found that blockade of either IL-1 or TNF attenuates the rise in PAI-1 mRNA (by 50%) but has no affect on the rise in PAI activity in plasma (Preliminary Studies, below). We have not yet examined the role of these mediators in the control of SPIP.

If SPIP proves central to the development of shock, and subsequent cell death, than a SPIP antagonist (receptor blocker, since antibodies will not cross the capillary wall), may offer a new therapeutic modality for patients in shock. However, an attempt to develop such a blocker (Specific Aim 3) requires identification of the active conformation of SPIP and of its receptor.

During the peroid of DAMD17-94-J-4002, we have shown that hemorrhagic shock elevates circulating PAI and stimulates PAI-1 transcription. We have developed a new method for quantifying mRNA and identified the cell types that are stimulated by hemorrhage to increase PAI-1 mRNA. We have determined that cytokines regulate the rise in PAI-1 mRNA after hemorrhage, but not secretion of PAI into plasma. We have determined that hemorrhage affects both transcription and translation of PAI-1 in liver. We believe that the active part of PAI-1 is the C-terminal 33aa fragment we call SPIP. SPIP acts to directly inhibit the Na/K ATPase and increases intracellular water and Na. We have recently purified SPIP from plasma on affinity columns containing PAI-1 antibodies or Na/K ATPase and partially purified SPIP elevates intracellular free calcium in cardiac myocytes.

Methods:

Conscious rat model: We use a modification of the chronically cannulated rat model originally described by Fagin *et al.* (34) and modified by Darlington *et al.* (20,21). Sprague-Dawley rats weighing 300-400g are anesthetized with pentobarbital sodium (50mg/kg, ip). Cannulas are placed in the femoral artery (Dural Plastics) and femoral vein (PE-50) for measurement of arterial blood pressure and heart rate and for injection of drugs and plasma sampling. The cannulas are tunneled under the skin of the back to exit at the back of the neck. A Dacron felt covered end of a stainless steel spring is connected to the back of the neck. The other end of the spring is connected to the top of the cage. Both cannulas traverse the spring to the top of the cage. All incisions are filled with Polysporin (Burroughs-Wellcome) before closure to prevent infection. Xylocaine jelly is topically applied to the incisions to desensitize the surgical areas. The rats are returned to the vivarium and given food and water ad libitum. The light cycle is 12hrs on and 12hrs off. The rats are allowed to recover for at least three days before experimentation.

Cannulas are flushed daily using heparin (1000U/ml). Experiments are performed in the vivarium. Neither the cages nor the rats are handled before the experiment. The cannulas are handled outside the cage so as not to disturb or unknowingly stress the animals prior to the experiment. The femoral artery cannula is connected to a COBE

pressure transducer (Cobe Laboratories, Inc., Lakewood CO) connected to a MICRO MED (Micro-Med Inc., Louisville, KY) blood pressure analyzer for recording arterial blood pressure and heart rate. Hemorrhage and blood samples for plasma constituents are taken from the femoral artery after the cannula is temporarily disconnected from the COBE transducer. The femoral vein cannula is used for infusion of drugs. Rats are fasted the night before by removing food, but not water.

A hemorrhage (25ml/kg of body weight in fasted rats, approximately 40% of blood volume) is performed in the morning 4 days after surgery. Blood samples (0.2ml) are taken over 0.15M sodium citrate, 180mg/ml of salicylic acid for PAI activity, and over 0.3M EDTA for ELISA. Plasma is separated by centrifugation and stored on ice. Some rats will be killed by decapitation 4hrs after hemorrhage and various organs harvested for determination of mRNA by RT-PCR.

***In Situ* Hybridization:** Male, Sprague-Dawley rats (300-350g) are anesthetized (50mg/kg) with pentobarbital sodium and perfused with 200ml ice cold 4% paraformaldehyde buffered in phosphate through the left ventricle of the heart. Organs are harvested and placed in 4% paraformaldehyde/phosphate buffer for 6hr followed by 25% sucrose (4°C) for at least 5days. Frozen sections (12μm) are placed on gelatin-coated glass slides in preparation for PAI-1 *in situ* hybridization as previously described (22). The sections are washed sequentially in PBS for 5 min, in 0.1M Tris HCl/0.05M EDTA with 0.1% Triton X-100, pH8.0 for 30min and in 0.1M Triethanolamine in 0.9% saline (titrated with HCl to pH 8.0) with 0.1% acetic anhydride for 10min. The slides are then washed in PBS before dehydration in 70, 95 and 100% ethanol, placed in chloroform (10 min) and rehydrated (100, 95, 70, 50% ethanol) into 2xSSC (8). The full length PAI-1 cDNA, generously donated by Dr. Thomas Gelehrter, was cut with Cel1 and BamH1 to produce a 534bp segment in the translated region of the gene. Sense and antisense riboprobes are synthesized from linearized plasmids (0.5 μg) using 10 units of T_3 and T_7 RNA polymerase, respectively, and 125 μCi of [^{35}S]UTP (1000Ci/mmol; DuPont-NEN, Boston, MA). Probe (2x10^6 cpm) diluted in 50 μl of hybridization buffer (50% formamide, 0.6 M NaCl, 10 mM Tris HCl, 1mM EDTA, 1xDenhardt's, 0.2 mg/ml of tRNA, 10% Dextran sulfate, 10 mM DTT, pH 7.4) is placed on each slide and a parafilm coverslip is placed on the tissue. The slides are incubated at 56°C overnight on 50% formamide soaked blotting paper in plastic incubation chambers wrapped in Saran Wrap to prevent dehydration. Following incubation, the sections are rinsed in 2xSSC to remove the coverslip and washed sequentially in 0.2xSSC with (1hr) and without (30min) 10μg/ml of RNase-A (Worthington Biochemicals, Freehold, NJ). Washing continues in 1xSSC, 0.5x SSC, 0.2xSSC (30min for all) and 0.2xSSC at 60°C for 1hr. Sections are dehydrated and then dipped in Kodak NTB-3 photographic emulsion (Eastman Kodak, Arlington VA) and exposed for 6-10 days, developed and counterstained with 0.4% methyl green. We have found that 6-10 days on emulsion gives a strong signal that is still on the linear portion of the curve.

Immunocytochemistry: Male, Sprague-Dawley rats (300-350g) are anesthetized (50mg/kg) with pentobarbital sodium and perfused with 200ml ice cold 4% paraformaldehyde buffered in phosphate. Organs are harvested and placed in 4%

paraformaldehyde/phosphate buffer for 6hr followed by 25% sucrose (4°C) for at least 5days. Frozen sections (12µm) are placed on gelatin-coated glass slides in preparation for PAI-1 immunocytochemistry. Sections are washed, sequentially, in PBS for 5 min, in 0.1M Tris HCl/0.05M EDTA with 0.1% Triton X-100, pH8.0 for 30min and in 0.1M Triethanolamine in 0.9% saline (titrated with HCl to pH 8.0) with 0.1% acetic anhydride for 10min. The slides are then washed in PBS before dehydration in 70, 95 and 100% ethanol, placed in chloroform (10 min) and rehydrated (100, 95, 70, 50% ethanol) into PBS. The sections are blocked with 10% normal goat serum (NGS), washed in PBS, blocked with 3% H_2O_2 in PBS and washed with PBS. The slides are placed, wet, on blotter paper soaked in PBS, in plastic incubation chambers. 100µl of 1ng/ml primary antibody (American Diagnostics, Inc.,) in 10% NGS/PBS is added to the tissue on the slides. The slides are incubated overnight at 4°C. Primary antisera are visualized using an avidin-biotin kit as per kit instructions (Vector Laboratories, Burlingame, CA). We are able to combine this procedure with *in situ* hybridization as shown in **Figure 8** below and as published (9,22-24)
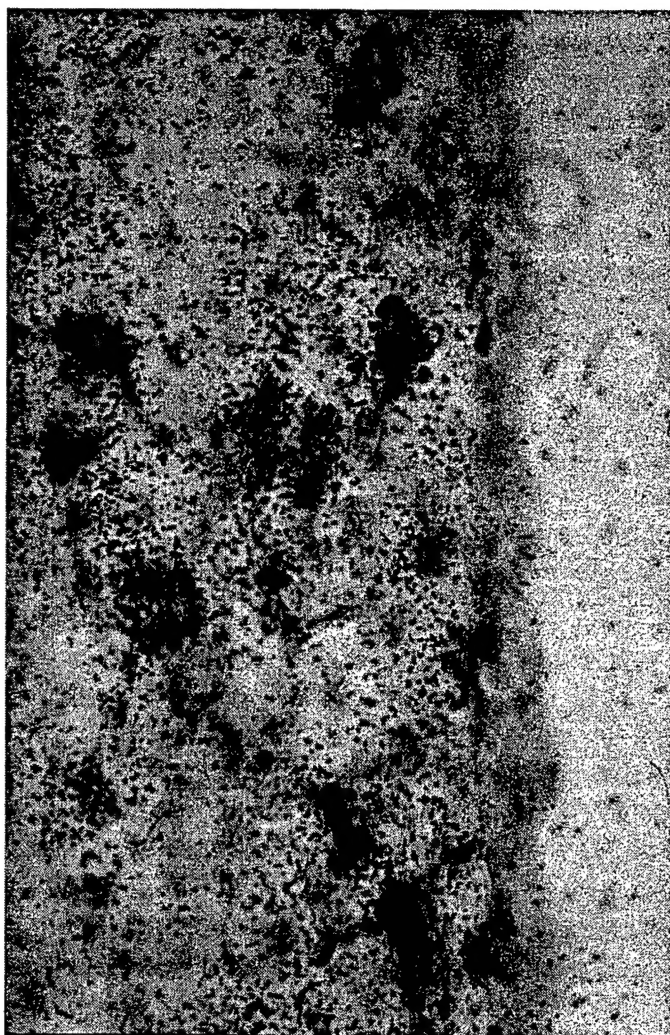

Figure 8. In situ hybridization for Regulate Endocrine Specific Protein-18 [silver grains] and immunocytochemistry for somatostatin in the paraventricular nucleus of the hypothalamus.

PAI Activity Assay: In the initial studies performed in this laboratory, plasma PAI activity was measured by Coatest PAI (Pharmacia Hepar, Franklin, OH) and was expressed in arbitrary units (AU), where 1 AU is the amount of PAI that inhibits 1 International Unit of tPA during a 10-min incubation at 22°C as described by the kit instructions. Inhibition is inferred from a loss of conversion of plasminogen to plasmin by tPA. Unfortunately, the Coatest does not take into account changes in plasma tPA which is elevated in septic shock (5,31,73) and hemorrhage (unpublished data). We have now developed a single step assay for plasma

PAI activity based on the principle that PAI-1 inhibits the conversion of a chromogenic substrate to p-nitroaniline (pNA) at 405 nm by uPA. The rate of appearance of pNA determines the slope of the reaction. PAI-1 inhibits uPA and depresses the rate of reaction, and therefore the slope. The difference between the slope of the control and PAI-1 samples represents the amount of PAI in the sample. Plasma samples are thawed on ice and diluted 10 fold with Tris-Triton buffer (0.1M Trizma Base (Sigma, St. Louis, MO) 5% v/v Triton X-100 (Pierce, Rockford, Ill) pH 8.8). 25µl of diluted plasma was added to each of 96 well microplate and 25µl uPA solution (4,000 IU/ml in Tris-Triton buffer, Calbiochem, La Jolla, CA). After incubation at 37°C for 30 min 50µl of artificial substrate (5 nM in DMSO, Calbiochem, La Jolla, CA) is added to each well and the initial rate of pNA production at 37°C is measured in a microplate reader. The assay is standardized with hrPAI-1. A manuscript describing this assay and comparing it to the Coatest has been submitted for publication.

RNA isolation: Organs are harvested and snap-frozen in liquid nitrogen. Total RNA is extracted by the acid guanidinium thiocyanate-phenol-chloroform method (19), and its concentration determined by measurement of sample absorbance at 260 nm.

Reverse transcription and Polymerase-Chain-Reaction (RT-PCR): A coupled reverse transcription-polymerase chain reaction (RT-PCR) is used to detect the changes of PAI-1 mRNA (43,53,81). The first strand of cDNA is synthesized in a 20 µl reaction volume containing 7 µg total cellular RNA, 400 units of Moloney murine leukemia virus reverse-transcriptase (Gibco BRL), 40 units of RNAse inhibitor, 10 mM dithiothreitol, 0.4 µg oligo dT, and all four deoxynucleotide triphosphates (0.5 mM each) in a reaction buffer of 50 mM Tris/HCl (pH 8.3), 75 mM KCl, and 3 mM $MgCl_2$. Each reaction mixture is heated to 65 °C for 5 min and placed on ice before adding MMLV-RT as the last step. The reverse transcription reaction is carried out for 90 min at 37 °C and then stopped on ice. The resulting cDNA samples are adjusted to PCR buffer conditions in a total volume of 50 µl, with 20 pmol of each primer and 1 U of Taq DNA polymerase (Promega). The primers for PAI-1 are 5'ATGAGATCAGTACTGCGGACGCCATCTTTG (335-364) and 5'GCACGGAGATGGTGCTACCATCAGACTTGT (667-638). According to Konkle *et al.*, the sequence of the amplified fragment is identical to the published sequence of the cDNA for rat PAI-1 (51). The primers for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are 5'TGATGACATCAAGAAGGTGGTGAAG (758-782) and 5'TCCTTGGAGGCCATGTAGGCCAT (997-975) (53). A three-temperature step PCR cycle program is carried out for the co-amplification of PAI-1 and GAPDH cDNA with an OmniGene Temperature Cycler (0.5 min annealing at 58°C, 1 min extension at 72°C, and 1 min denaturation at 94°C). The lengths of the PCR products are checked by electrophoresis with 1 Kb DNA Ladder (Gibco, BRL) in a 1 % agarose gel. PCR reliability is estimated by amplification of equal amounts (0.2 µg) of reverse-transcribed RNA obtained from the liver of endotoxin-treated rats (positive controls). Twenty-six to 36 PCR cycles were run to determine the dependency of the amount of PCR products upon cycle numbers. Linearity between the amount of amplified cDNA and that of applied RNA was ascertained in the pre-plateau exponential phase. With these

preliminary data, all PCR experiments will be performed using 29 cycles.

We constructed heterologous competitors for PAI-1 and GAPDH containing identical primer sites. The quality and amount of RNA, not controlled for using a RNA competitor, was standardized by constructing a competitor to GAPDH, an internal control housekeeping gene. We proved the competitors and target sequences were amplified with equal efficiencies using identical primer pairs, thus the initial ratio of target to competitor remained constant throughout the reaction allowing us to determine the original amount of target present. The competitor and wild type message amplified with equal efficiency for each competitor for both the GAPDH and PAI-1 reactions. The amount of target in the sample was determined from the amount of competitor that yields equal amounts of target and competitor PCR products. Use of competitors effectively controls for tube-to-tube variation in amplification efficiency. PCR products are rapidly separated and quantitated by capillary electrophoresis (CE) coupled with a laser that excites a fluorescent dye specific for dsDNA. Addition of a 200 bp standard controls for CE nanoliter injections. The peak area value for PAI-1mRNA at each time point is normalize for GAPDH mRNA as follws: peak area of PAI-1 mRNA (Absorption units x min) x mean peak area of GAPDH mRNA (AU x min). Capillary electrophoresis can quantify fg concentrations from as little as 1ul of PCR sample providing reproducible data in 7 minutes. This technique represents a considerable advance in that it controls for the efficiency of the PCR reaction and provides reproducible data in a far shorter time than using conventional techniques (37,38).

Preparation of Adult Cardiac Myocytes. Rat ventricular myocytes are isolated after Langendorff perfusion with low Ca^{2+}, collagenase (type B: Boehringer Mannheim), and protease (Type XIV; Signa). Cells are suspended in 137mM NaCl, 5mM KCl, 15mM dextrose, 1.3mM $MgSO_4$, 20mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) at pH7.4 (NaOH) and 1mM Ca^{2+}. Cells are stored at 37°C until experiment. For measurement of intracellular calcium, myocytes are loaded with Fluo-3 (Molecular Probes) by incubation for 15min with 5μM Fluo-3 acetoxymethyl ester. For measurement of intracellular sodium, myocytes are loaded with SBFI (Molecular Probes) by incubation for 15min with 5μM SBFI acetoxymethyl ester. The myocytes are placed in a bath on the fluorescence microscope and superfused (0.6ml/min) with control buffer (125mM NaCl, 5mM KCl, 20mM HEPES, 20mM glucose, 0.8mM $MgSO_4$, 1mM NaH_2PO4 in 1% DMSO at pH7.4 adjusted with NaOH) at 30°C. Myocytes are field stimulated at 1Hz using 5ms pulses with a magnitude 1.5 times threshold. $[Ca^{2+}]i$ and $[Na^+]$ transients are measured on a Nikon Diaphot using a fluorescence detection system (Photon Technology International (PTI), South Brunswick, NJ, USA). A 75W xenon lamp with a dichroic longpass (DCLP) mirror is used. For Flou-3, the excitation wavelength is 488nm and emission wavelength is 510nm; for SBFI, the excitation wavelength is 340nm and emission wavelength is 505nm. $[Ca^{2+}]i$ transients are reported as relative fluorescence, or *F/Fo*, where *F* is fluorescence emission and *Fo* is the resting fluorescence recorded at the start of the experiment as previously described (27,28,49).

In Situ RT-PCR

Tissue sections were fixed and deproteinated as above. DNA digestion was

performed in 10 mM Tris-HCl, pH7.4, 10 mM $MgCl_2$, and 1 U/ml RNase-free DNase (Boehringer Mannheim) overnight at 37°C. After washing for 1 min in diethylpyrocarbonate-treated water and for 1 min in 100% ethanol, reverse-transcription was performed at 42°C for 45 min in 50 mM Tris-HCl, pH8.3, 75 mM KCl, 5.5 mM $MgCl_2$, 1 mM 3'primer, 2 U/ml RNase inhibitor, 0.5 mM dNTPs, 10 mM dithiothreitol, and 2 U/ml MMLV-RT. To prevent evaporation, the solution was covered with a coverslip, anchored with a drop of nail polish and overlaid with mineral oil (62). After the section was washed in xylene for 3 min and in 100% ethanol for 3 min, PCR was performed using 0.4 mM of 5'primer and 3'primer, 0.1 U/ml Taq DNA polymerase, 50 mM KCl, 10 mM Tris-HCl, pH9.0, 0.1% Triton X-100, 4.5 mM $MgCl_2$, and 0.2 mM dNTPs. The solution was covered again with a coverslip, anchored with a drop of nail polish and overlaid with mineral oil. A two-temperature step PCR cycle program was carried out with an OmniGene Temperature Cycler (2 min annealing and extension at 72°C, and 1 min denaturation at 94 °C). Livers of endotoxin-treated rats (positive controls) were analyzed after 6, 12, 18, and 24 PCR cycles. Twelve cycles were found to be optimal for analysis, and all further *in situ* RT-PCR studies were performed at 12 cycles. Liver sections from control, hemorrhage and some endotoxin rats were run side by side for direct comparison. After PCR, the sections were washed in xylene for 3 min and in 100% ethanol for 3 min, denatured at 96°C for 7 min, quickly chilled on ice, and then hybridized with the digoxigenin-labeled probe at 42°C for 2 h in 5x SSC, 0.1% N-lauroylsarcosine, 0.02% SDS, 1.0% (wt/vol) casein, and 25 pmol/ml digoxigenin-labeled probe. After hybridization, the sections were washed twice in 2x SSC, 0.1% SDS for 5 min each, and twice in 0.5x SSC, 0.1% SDS for 15 min each. Digoxigenin-labeled nucleotide was detected as described above. As a methodological control, the sections were analyzed with a modified *in situ* RT-PCR, where either the RT step was omitted, the RT step was preceded by a 2 h-incubation in 100 mg/ml DNase-free RNase (Boehringer Mannheim) at 37°C, or the irrelevant probe was used for hybridization.

In this study, the probe was complimentary to the internal sequence of the expected PCR product to avoid false positive signals due to amplification of genomic DNA (50). False positive signals resulting from the amplification of the endogenous DNA were excluded by performing control experiments that 1) omitted the RT step or 2) preceded the RT step with RNA digestion.

Results and Discussion

<u>Hemorrhage elevates PAI activity in plasma</u>
We have found that PAI activity as measured by Coatest (Pharmacia Hepar, Franklin, OH) is significantly elevated 0.5, 1, 2, 4, 6, and 8h after 20ml/kg hemorrhage in conscious rats as compared to pre-hemorrhage levels (**Figure 1**) and the responses were significantly different from those of the non-hemorrhage controls.
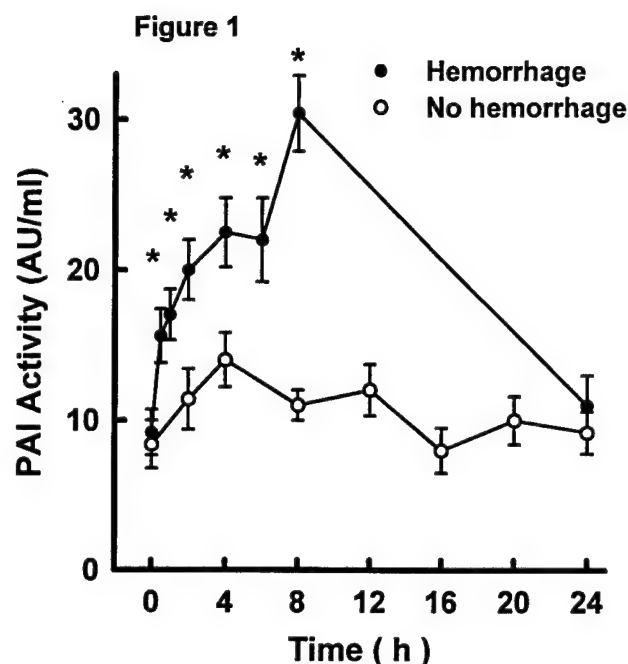
Figure 1

Figure 1. Plasma PAI activity rises after hemorrhage in conscious rats. * $P<0.05$ compared to non-hemorrhage controls by Newman-Kuels Post Hoc test following 2-Way ANOVA. Hemorrhage group n=8, non-hemorrhage group n=6.

PAI-1 is the major physiologic inhibitor of both tissue type and urokinase type plasminogen activators (52,89). PAI activity is the ability of PAI to inhibit the action of plasminogen activators. We use two enzyme assays to measure PAI activity in plasma, one that uses tPA (Coatest, Pharmacia Hepar, Franklin,OH) and one that uses uPA (conversion of substrate specific for uPA to a colored product). These assays can only detect the presence of active PAI, not the latent, substrate or SPIP forms.

SPIP and PAI-1 ELISA: SPIP and PAI-1 antigen is measured with a direct ELISA using a polyclonal rabbit anti-rat SPIP IgG directed against a fragment of SPIP (10aa - RSFLFVVRHN - near the N-terminus, developed by Anaspec, Inc.). The ELISA is visualized with a peroxidase-conjugated goat affinity purified secondary antibody to rabbit IgG (Organon Teknika Corp., Durham, NC). This 1° antibody cross-reacts with both PAI-1 and SPIP. Therefore, the ELISA will measure both PAI-1 and SPIP. To separate SPIP for PAI-1, plasma is run over an anhydro-urokinase column (87) that binds PAI-1 but not SPIP. The ELISA for SPIP is run on the flow through. The ELISA for PAI-1 is run on the arginine eluent. We use hrPAI-1 generously donated by Dr. Harry L. Walton, Du Pont Merck Pharmaceutical Company, to standardize the total PAI-1 assay, and the synthetic SPIP produced by the University of Maryland School of Medicine Biopolymer Laboratory to standardize the SPIP assay.

Additional antibodies specifically directed against the amino acids 10-19 and 24-33 of SPIP are currently under development by Hazelton, Inc. The antigens were made synthetically at the University of Maryland Biopolymer Laboratory by coupling multiple copies of the 10-19 or 24-33 fragments to KLH.

Measurement of mRNA by RT-PCR.
We have developed an improved method for measuring PAI-1 mRNA in tissue homogenates. After extraction of RNA from the tissue, the mRNA is converted to cDNA by reverse transcription and the cDNA is amplified by PCR. The PCR product of PAI-1 (cDNA) is separated by capillary electrophoresis and measured by laser spectroscopy.

We use the endogenous gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to control for variations in PCR efficiency (89). This method now being used by a number of laboratories, but is relatively new. Recently, we have incorporated the use of internal competitors to control for PCR inefficiency (37,38). The competitor for PAI-1 has the same primer hybridization sites on the ends of the gene with nonsense code in-between. The competitor is separated from the wild type by capillary electrophoresis because of it different molecular weight. Both wild type PAI-1 and competitor cDNA are quantified by laser spectroscopy. The amount of wild type PAI-1 is quantified from the ratio of competitor to wild type cDNAs and the amount of competitor cDNA added (37,38). At present, we are extending this method to correct for variation in reverse transcription by using competitor mRNA instead of cDNA.

Hemorrhage elevates PAI-1 mRNA

We found that both hemorrhage and sepsis lead to elevated PAI-1 mRNA above control in homogenates of liver (**Figure 2a**). PAI-1 mRNA rose significantly in liver homogenates 1, 2, 4, and 6 h after 20 ml/kg hemorrhage. The increase peaked at 4 h after hemorrhage and then fell to near control levels by 24 h (**Figure 2b**). Fifteen ml/kg hemorrhage led to a significantly smaller increase in PAI-1 mRNA in liver. PAI-1 mRNA was also significantly elevated above pre-hemorrhage levels in lung, heart, and kidney 4 h after 20 ml/kg hemorrhage (**Figure 2c**). In this study endotoxin was also used as a positive control. These data indicate that hemorrhage rapidly increase in PAI-1 mRNA in a variety of organs. This finding is significant in that it shows that hemorrhage causes an increase in PAI-1 mRNA of a similar magnitude to endotoxin, a potent and clinically important inducer of PAI-1 mRNA expression. Additionally, the increase in PAI-1 mRNA appears to be dependent on the magnitude of blood loss (89).
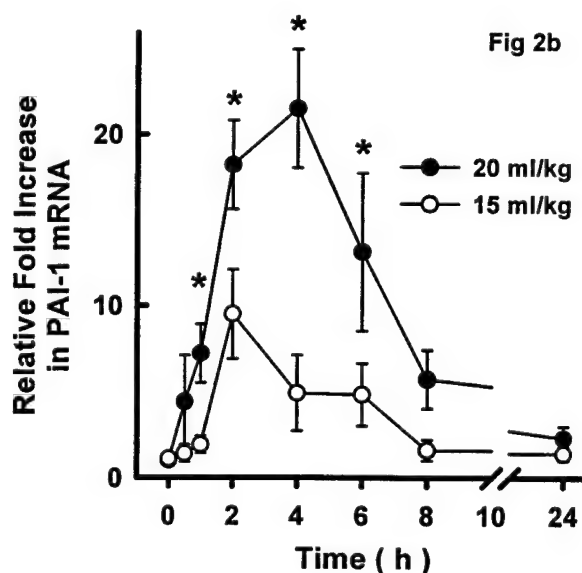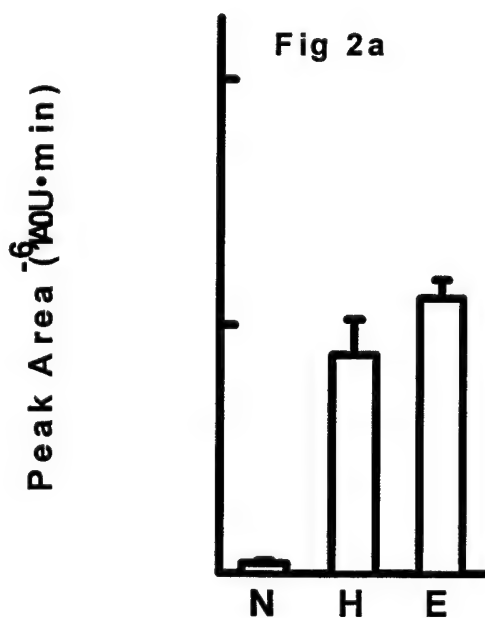

Fig 2a

Fig 2b

Figure 2a. Affect of hemorrhage (H, 20ml/kg) and sepsis (E, *Escherichia Coli* lipopolysaccharide, 300 µg/kg) as compared to normal (N) on PAI-1 mRNA in conscious rats (n=5/each) as measured by HPLC after reverse transcription PCR. H and E are statistically different from N by Student's t-Test.

Figure 2b. Rise in PAI-1 mRNA in homogenates of liver after 15 (n=4/time point) and 20ml/kg hemorrhage (n=5/time point) in conscious rats as measured by HPLC after reverse transcription PCR. *=P<0.05 as compared to 15ml/kg hemorrhage by Newman-Keuls post hoc test after 2-Way ANOVA.

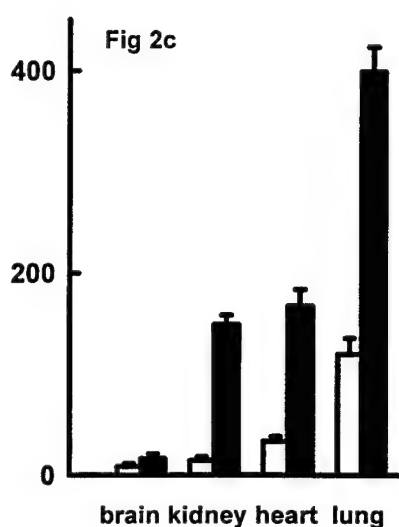Fig 2c

brain kidney heart lung

Figure 2c. Rise in PAI-1 mRNA in homogenates of brain, kidney heart and lung 4hr after 20ml/kg hemorrhage (n=5rats).

Hemorrhage stimulates transcription of PAI-1 mRNA in endothelial and mesothelial cells.

To determine the cell types stimulated by shock to produce PAI-1, we have used *in situ* PCR. In liver, hemorrhage led to an elevation in PAI-1 mRNA in vascular endothelial cells and mesothelial cells, but not in the hepatocytes **(Figure 3).** The difference between control and bled rats is striking using *in situ* PCR. PAI-1 mRNA is undetectable without hemorrhage (89).
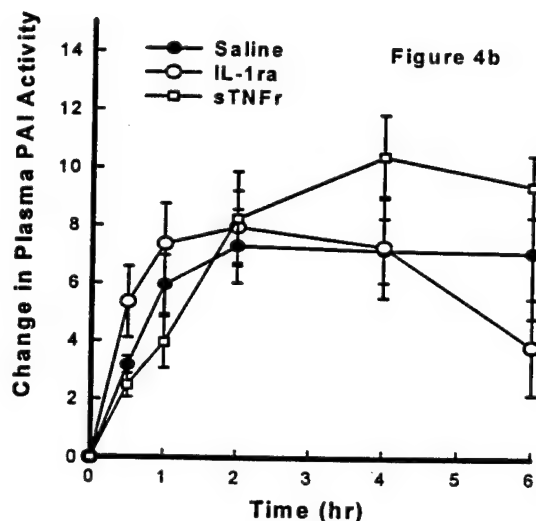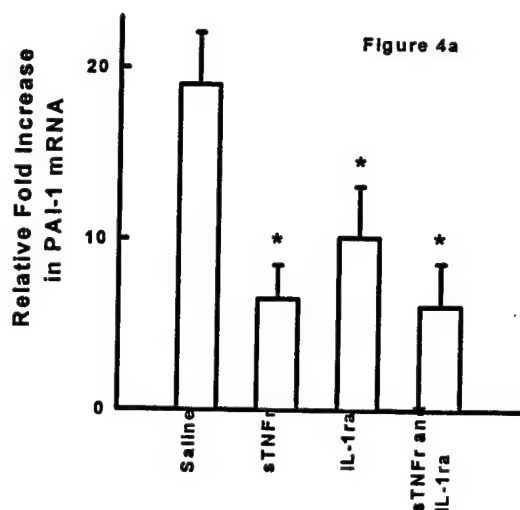
Figure 3

In situ PCR showing elevated PAI-1 mRNA signal in Mesothelial and Endothelial Cells in the liver of rats that have been hemorrhaged.
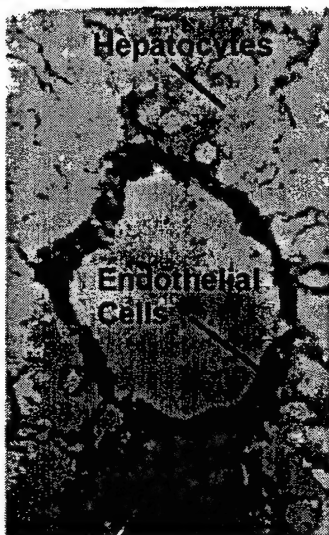
We used *in situ* PCR because in situ hybridization with 45mer end-labeled oligoprobes was not sensitive enough to visualize signal. In situ PCR uses the RT-PCR reaction on slices of tissue mounted on glass microscope slides to achieve a 50-fold amplification of mRNA thereby allowing the presence of small quantities of mRNA to be visualized in various morphologically distinct cell types (89). However, the technique has its problems. The thermocycling of the tissue does not allow for good morphology, thereby making morphological determination of cell types difficult. To circumvent this problem, we have received the full-length cDNA for PAI-1 (generously donated by Dr. Thomas D. Gelehrter, Departments of Human Genetic and Internal Medicine, University of Michigan Medical School). A 534bp segment in the translated region of the gene has been cut out and placed in a Bluescript vector. Sense and antisense riboprobes are synthesized from the Bluescript linearized either T3 or T7 polymerase. Wash conditions for the riboprobes have been determined. In the last month, we found PAI-1mRNA elevated in patches of liver after hemorrhage or sepsis.

Figure 4a

Figure 4b

Mediation of PAI-1 transcription and secretion by cytokines:

Knowing that PAI-1 mRNA and protein are elevated in vascular endothelial cells and mesothelial cells (52,72,84,85,88) by TNF and IL-1, AND that hemorrhage elevates both TNF and IL-1, we set out to determine if TNF and/or IL-1 mediate the rise in PAI-1 mRNA and plasma PAI activity after hemorrhage. Using the soluble TNF receptor and IL-1 receptor antagonist (Amgen Inc.), we found that blockade of TNF and/or IL-1 severely attenuated the rise in PAI-1 mRNA to 20ml/kg hemorrhage (**Figure 4a**).

However, blockade of TNF or IL-1 had no effect on the rise in plasma PAI activity (**Figure 4b**). This suggest that the cytokines mediate, in part, the rise in PAI mRNA after hemorrhage, but not the secretion.

Figure 4a. PAI-1 mRNA in liver homogenates taken 4hrs after 20ml/kg hemorrhage. Conscious rats were treated with either 1) 0.2 ml saline (iv), 2) 1.5mg/kg of soluble TNF receptor (Amgen Boulder Inc., Boulder CO) in 0.2ml saline, 3) 30mg/kg IL-1 receptor antagonist (IL-1ra, Amgen Boulder Inc.) in 0.2ml saline or 4) both 15 min before and 2 h after 20 ml/kg hemorrhage. n=5rats/group *=P< 0.05 compared to saline control by Newman-Keuls post hoc test after 1-way ANOVA.

Figure 4b. PAI activity in plasma after 20ml/kg hemorrhage in conscious rats treated with either 1) 0.2 ml saline (iv), 2) 1.5mg/kg of soluble TNF receptor in 0.2ml saline or, 3) 30mg/kg IL-1 receptor antagonist in 0.2ml saline. n=8rats/group. There was no significant difference between groups (2-Way ANOVA).

A similar finding is shown for endotoxin shock (31). Conscious rats given LPS showed a large rise in plasma PAI activity that was not affected by TNF blockade. However, TNF blockade attenuated that rise in PAI-1 mRNA in liver homogenates. This suggests that these cytokines play a role in the rise in PAI-1 mRNA transcription during hemorrhage or

sepsis, but not in the secretion or appearance of PAI in plasma.

Regulation of PAI release by inhibitors of transcription and translation.
To determine if the hemorrhage-induced elevation in plasma PAI activity is dependent on *de novo* synthesis of PAI-1 protein, we pretreated conscious rats with cycloheximide to block translation of mRNA into protein. As shown in **Figure 5,** cycloheximide does not totally block the increases in plasma PAI activity seen after hemorrhage. However, there may be some attenuation of the early response (0 to 2 hrs). The effectiveness of the blockage of translation was checked by assaying for plasma levels of adrenocorticotropin (ACTH) in the same animals by radioimmunoassay. ACTH, which is elevated after hemorrhage (31), failed to increase in response to hemorrhage after pretreatment with cycloheximide (data not shown). This indicates that the cycloheximide was effective in preventing translation of mRNA.
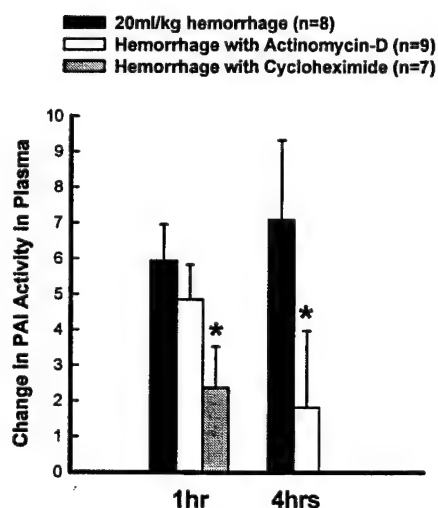

Figure 5. Effect of cycloheximide or actinomycin-D treatment on the hemorrhage induced rise in plasma PAI activity in conscious rats. *=$P<0.05$ by Newman-Keuls post hoc test after ANOVA.

To determine if the hemorrhage-induced elevation in plasma PAI activity is dependent on an elevation in PAI-1 mRNA, we pretreatment conscious rats with actinomycin-D to prevent transcription. We found that actinomycin-D does not prevent the early (0-2 hr) increase in plasma PAI activity after hemorrhage but it may attenuate the later sustained rise in activity (**Figure 5**). In these experiments, we measured PAI-1 mRNA following hemorrhage to check the effectiveness of the blockade of transcription. The increase in PAI-1 mRNA was prevented by actinomycin-D (data not shown), indicating that the treatment was effective in preventing transcription. These data along with those of the above experiment suggest that the early rise in plasma PAI activity seen after hemorrhage is dependent on translation of preformed mRNA to protein and not on transcription of DNA to mRNA. However, the late sustained rise is, in part, dependent on transcription of new message.

In situ hybridization and immunohistochemistry for PAI-1. We now have preliminary evidence that shows PAI-1 mRNA elevated after 20ml/kg hemorrhage in patches of cells in the liver. Sepsis causes the same elevation, but to a greater extent. In the future, we will immunostain in combination with *in situ* hybridization to determine which cell types, besides endothelial and mesothelial cells, in liver and other tissues are stimulated by hemorrhage and sepsis. The combination of *in situ* and immunocytochemistry for Endothelin and Von Willebrand Factor will help us determine if PAI-1 mRNA is elevate in endothelial cells. We have been successful combining *in situ* with

immunocytochemistry (21-24) and expect few difficulties identifying the cell types with elevated PAI-1 mRNA.

SPIP inhibits the Na^+/K^+ ATPase in a dose dependent manner.

Vesicles containing Na^+/K^+ ATPase were prepared from beef brain (32). The mitochondrial ATPases were inhibited with sodium azide. Calcium ATPase was inhibited by holding the extra-vesicle Ca^{++} concentration at 150nM with EGTA. Under these conditions, approximately 85-100% of phosphate released from added ATP (measured colorimetrically at 650nm) was inhibited by superphysiologic concentrations (500μM) of ouabain indicative of Na pump activity. The calcium concentration was set high enough to permit actions through membrane receptors and G-proteins so that direct action on the sodium pump cannot be inferred. Under these conditions, the addition of increasing concentrations of synthetic SPIP showed progressive inhibition of the Na^+/K^+ ATPase activity with inhibition equivalent to that of ouabain at 1nM (**Figure 6**). However, this action requires the presence of 4μM ferric iron. Similarly, SPIP directly inhibits purified soluble Na^+/K^+ ATPase prepared from dog or rabbit kidney in a dose response manner. Again, maximum activity was observed at 1nM and similar concentrations of ferric iron was required for any significant inhibition (**Figure 6b**).

<p align="center">Figure 6a   Figure 6b</p>

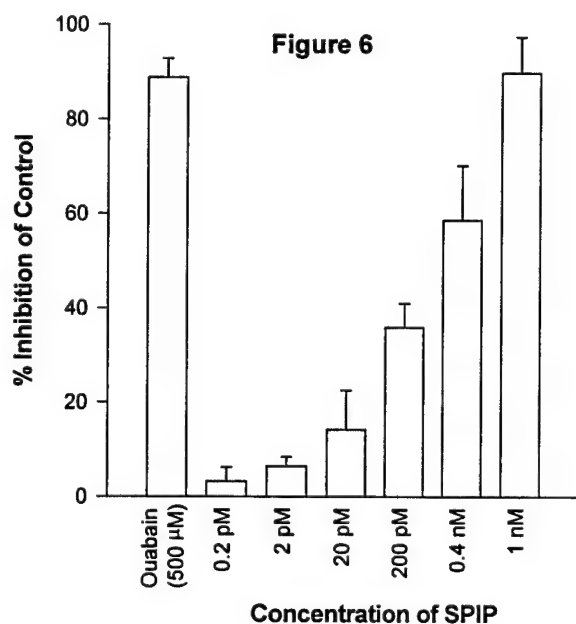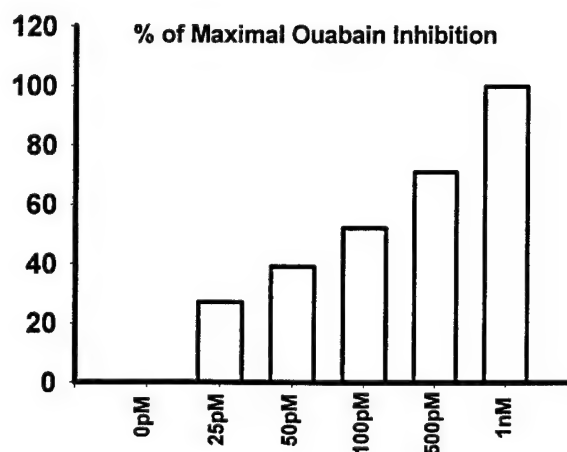**Figure 6a**. SPIP inhibits membrane vesicle Na^+/K^+ ATPase in a dose response manner. Effect of ouabain is shown at left. n=9/concentration run in duplicates.

Figure 6b. SPIP inhibits purified soluble dog kidney Na^+/K^+ ATPase in a dose response manner. Values represent the mean of duplicates.

The concentration of SPIP in shock plasma is sufficient to account for the observed inhibition of Na^+/K^+-ATPase.

In preliminary studies, plasma from shock rats was analyzed with the ELISA described below. SPIP was separated from plasma PAI-1 using anhydo-urokinase columns prior to assay (PAI-1 binds anhydro-urokinase, SPIP does not, ref 87). SPIP concentrations were derived from a standard curve constructed with varying amounts of synthetic SPIP. In 3 rats, plasma SPIP increased 4-5 fold above controls after hemorrhage, reaching a final concentration of approximately 1.8nM. This concentration is sufficient to account for as much inhibition of Na^+/K^+ ATPase as is seen with supramaximal concentrations of ouabain. Furthermore, this amount of inhibition can account for the increases in cell sodium and water that have been observed in hemorrhagic shock (14).

SPIP increases intracellular water and sodium.
Intracellular water was measured as the difference between ^3H water space and ^{14}C mannitol space. SPIP partially purified from shock plasma increased intracellular water 9% in rat red blood cells, 22% in rat muscle cells, 11% in dog red cells and 31% in dog white blood cells (10). In recent experiments, synthetic SPIP has been shown to produce similar changes. In a few experiments, we have examined the effects of synthetic SPIP on intracellular sodium. Cardiac myocytes were loaded with the sodium sensitive dye SBFI. SPIP caused intracellular sodium to increase in a manner similar to ouabain. This suggests that the changes in transmembrane potential observed in hemorrhagic shock and produced by SPIP are accompanied by a movement of water and sodium from the interstitium to cells and may account for the inability to restore blood volume after large hemorrhage.

Plasma purified on PAI-1 antibody or Na^+/K^+ ATPase columns retains SPIP activity.
Plasma from shocked rats was passed over G25-sephadex, twice. The eluate was free of both albumin and showed no PAI activity. Only low molecular weight peptides were found on SDS-PAGE gel. The eluate completely inhibited Na^+/K^+ ATPase in our assay. The eluate was passed over affinity columns in which either antibody to PAI-1 or rabbit Na^+/K^+ ATPase (Sigma) was bound to agarose beads. Each column removed all ATPase inhibitory activity. The eluate contained the activity and thus appears to contain a derivative of PAI-1 that binds to the sodium pump.

Cloning and Expression of SPIP
We have cloned SPIP by inserting the SPIP cDNA into a pET32a vector (Novagen) between the BamHI-1 and NcoI restriction sites. The cDNA was engineered to contain these restriction sites plus a stop codon. The SPIP pET32a vector encodes a thioredoxin fusion protein, a poly-histidine site and SPIP. *E. Coli* BL21 DE3 and BL21 PlysS (Novagen) were transformed with SPIP pET32a. 22 surviving colonies were cloned and showed the correct sequence. Expression was induced by 0.1mM IPTG incubated for 2hrs at 37°C. The protein was separated from the homogenate over a Nickel column that binds the poly-histidine site. SPIP-fusion protein was eluted off column with 1.0M imidazole. We estimate from SDS-PAGE that greater than 80% of the total protein expressed was SPIP-fusion protein. The fusion protein was cleaved with enterokinase leaving an N-terminal alanine on SPIP (ala-SPIP).

<u>Resuscitation with whole blood:</u>
Preliminary experiments show that the hemorrhage-induced rise in plasma PAI activity in conscious rats is not attenuated after resuscitation with whole blood. However, resuscitation does lower the ability of plasma to depolarize cells *in vitro* (11). This suggests that resuscitation may inhibit SPIP. Experiments outlined in the current proposal (Aim 1), we have not found inhibition of PAI-1 with crystaloid resuscitation. We do not have results to draw a **conclusion** about SPIP.

<u>SPIP acitivity requires Fe3+:</u>
We have shown that Fe3+ is required for formation of SPIP. In the absence of Fe3+ partially purified plasma only weakly inhibits Na,K-ATPase (20%). *In vitro* the requirement for Fe3+ may be met by 100mM NH_4^+ ion. We have also determined that the interaction of SPIP with its receptor on the Na,K/ATPase requires micromolar Fe3+, a requirement not satisfied by NH_4^+. We have determined that iron chelation with desferrioxamine inhibits both activity as well as its receptor binding. Desferrioxamine has previously been shown to be protective in shock (26a). Previously, we had shown that MgATP inhibits formation of depolarizing activity in plasma.

Conclusions:

In summary, we have found that hemorrhagic shock significantly elevated circulating PAI-1 and PAI-1 mRNA in conscious rats. This appears to be dose **dependent.** We have developed an improved method for measuring PAI-1 mRNA in tissue homogenates using RT-PCR. We found that both hemorrhage and sepsis lead to elevated PAI-1 mRNA above control in homogenates of liver, rising significantly in liver homogenates 1, 2, 4, and 6 h after 20 ml/kg hemorrhage and falling to near control levels by 24 h. Fifteen ml/kg hemorrhage led to a significantly smaller increase in PAI-1 mRNA in liver. PAI-1 mRNA was also significantly elevated above pre-hemorrhage levels in lung, heart, and kidney 4h after 20 ml/kg hemorrhage. These data suggest that hemorrhage activates transcription of PAI-1 mRNA in a variety of organs. Additionally, the increase in PAI-1 mRNA appears to be dependent on the magnitude of blood loss.

Focusing on liver, we found that hemorrhage led to an elevation in PAI-1 mRNA in vascular endothelial cells and mesothelial cells, but not in the hepatocytes. All three cell types expresses PAI-1, however, **hemorrhage** does not activate all of them. Also, blockade of TNF and/or IL-1 severely attenuated the rise in PAI-1 mRNA to hemorrhage in liver, but had no effect on the rise in circulating PAI activity, suggesting that cytokines partially mediate the rise in transcription, but not the secretion of PAI-1 after hemorrhage.

Knowing that hemorrhage changes expression of PAI-1, we determined if the hemorrhage-induced elevation in plasma PAI activity is dependent on *de novo* synthesis of PAI-1 protein. Conscious rats were pretreated with cycloheximide to block translation of mRNA into protein. Cycloheximide partially blocked the increases in plasma PAI activity seen after hemorrhage. These data along with those of the above experiment suggest that the early rise in plasma PAI activity seen after hemorrhage is dependent on translation of preformed mRNA to protein and not on transcription of DNA to mRNA. However, the late sustained rise is, in part, dependent on transcription of new message

Recent evidence shows PAI-1 mRNA is elevated after 20ml/kg hemorrhage in patches of cells in the liver (in situ hybridization). We also found that sepsis causes the same elevation, only to a greater extent. Because this method of in situ allows for better morphology, future experiments will allow us to pinpoint the cell types that are sensitive to hemorrhage-induced changes in PAI-1 expression.

We have recently found that SPIP (synthesized by Dr. N. Ambulos of the Biopolymer Laboratory at the University of Maryland School of Medicine) inhibits the Na^+/K^+ ATPase in membrane vesicles in a dose dependent manner with activity in nM concentration as great as that of ouabain in μM concentrations. Because of this finding, we strongly believe that the SPIP is the humoral agent in plasma that causes depolarization of cells by directly or indirectly inhibiting the Na^+/K^+ ATPase pump and thereby leads to the early pathophysiological features of shock described by Shires (75,76).

It has been reported that plasma PAI activity is elevated after septic shock in man (64,68) and pig (77), anaphylactic shock in rabbits (74) and cardiac by-pass surgery in man (16). We have now shown that both PAI activity in plasma and PAI-1 mRNA in liver are elevated following both hemorrhage and sepsis in rat liver (89). We have also shown that hemorrhage and sepsis stimulates transcription of PAI-1 in endothelial and mesothelial cells of rat liver (88). These findings raise questions about what humoral factors mediate the rise in PAI-1 transcription and secretion after hemorrhage. TNF, IL-1, IL-6, angiotensin II, Transforming Growth Factor-β and glucocorticoids, all have been shown to stimulate synthesis of PAI 1 and PAI-1 mRNA in endothelial and mesothelial cells, and hepatocytes (26,35,39,41,42,45,46,55-57,60,67,72,78,90). Furthermore, many of these substances are elevated after hemorrhage and shock (20,21,63,80,82). We have set out to determine which humoral agents mediate the rise in PAI-1 mRNA and secretion after hemorrhage. We have recently found that blockade of either IL-1 and TNF attenuates the rise in PAI-1 mRNA (by 50%) but has no affect on the rise in PAI activity in plasma (Preliminary Studies, below). We have not yet examined the role of these mediators in the control of SPIP.

SPIP directly inhibits purified soluble Na^+/K^+ ATPase prepared from dog or rabbit kidney in a dose response manner and maximum activity occurs at 1nM making SPIP a potential physiologic controller of the Na^+/K^+ punp. SPIP partially purified from shock plasma increased intracellular water 9% in rat red blood cells, 22% in rat muscle cells, 11% in dog red cells and 31% in dog white blood cells (10). In recent experiments, synthetic SPIP has been shown to produce similar changes. In a few experiments, we have examined the effects of synthetic SPIP on intracellular sodium. Cardiac myocytes were loaded with the sodium sensitive dye SBFI. SPIP caused intracellular sodium to increase in a manner similar to ouabain. This suggests that the changes in transmembrane potential observed in hemorrhagic shock and produced by SPIP are accompanied by a movement of water and sodium from the interstitium to cells and may account for the inability to restore blood volume after large hemorrhage.

Finally, preliminary experiments show that the hemorrhage-induced rise in plasma PAI activity in conscious rats is not attenuated after resuscitation with whole blood. However, resuscitation does lower the ability of plasma to depolarize cells *in vitro* (11). This suggests that resuscitation may inhibit SPIP.

If SPIP proves central to the development of shock, and subsequent cell death, than a SPIP antagonist (receptor blocker, since antibodies will not cross the capillary wall), may offer a new therapeutic modality for patients in shock. However, an attempt to develop such a blocker is premature without further experimentation.

References:

1. Adolph EF, Gerbasi MJ, Lepore MJ. The rate of entrance of fluid into the blood in hemorrhage. Am. J. Physiol. 104:502-517, 1933.
2. Aertgeerts K, De Bondt HL, De Ranter C. A model of the reactive form of plasminogen activator inhibitor-1. J. Struct. Biol. 113:239-245, 1994.
3. Andreasen PA, Riccio A, Welinder KG, Douglas R, Sartorio R, Nielsen LS, Oppenheimer C, Blasi F, Dano K. Plasminogen activator inhibitor type-1: reactive center and amino-terminal heterogeneity determined by protein and cDNA sequencing. FEBS Lett. 209:213-217, 1986.
4. Arango A, Illner H, Shires GT. Role of ischemia in the induction of changes in cell membrane during hemorrhagic shock. J. Surg. Res. 20:473-476, 1976.
5. Arndt A, Murphy P, Hart DA. Human HuH-7 hepatoma cell express urokinase and plasminogen activator inhibitor-1: identification, characterization and regulation by inflammatory mediators. Biochim. Biophys. Acta, 1138:149-156, 1992
6. Audenaert A-M, Knockaert I, Collen D, Declerck PJ. Conversion of plasminogen activator inhibitor-1 from inhibitor to substate by point mutations in the reactive-site loop. J. Biol. Chem. 269:19559-19564, 1994.
7. Baue AE, Tragus ET, Parkin WM. Effects of increased osmolality and correction of acidosis on blood flow and oxygen consumption in hemorrhagic shock. J. Surg. Res. 7:349-356, 1974.
8. Bhat RV, Baraban JM, Johnson RC, Eipper BA, Mains RE. High levels of expression of the tumor suppresser gene APC during development of the rat CNS. J. Neurosci 14:3059-3071, 1994.
9. Bloomquist, BT, Darlington DN, Mains RE, Eipper BA. RESP18, A novel neuroendocrine-specific transcript, and four other transcripts, are regulated in parallel with POMC in melanotropes. Journal of Biological Chemistry, 269:9113-9122,1994
10. Borchelt, BD, Wright PA, Evans JA, Gann DS. Cell swelling and depolarization in hemorrhagic shock. J. Trauma 39:187-194, 1995.
11. Boulanger BR, Evans JA, Lilly MP, Shurtleff DM, Williams JC, Gann DS. A circulating protein that depolarizes cells increases after hemorrhage in dogs. J. Trauma. 34:591-599, 1993.
12. Boyd DR, Mansberger AR. Serum water and osmolal changes in hemorrhagic shock: experimental and clinical studies. Am. Surg. 34:744-749, 1968.
13. Byrnes GJ, Pirkle JC Jr. Gann DS. Cardiovascular stabilization after hemorrhage depends upon restitution of blood volume. J. Trauma. 18:623-627, 1978.
14. Carlson DE, Kligman MD, Gann DS. Impairment of blood volume restitution after large hemorrhage: a mathematical model. Am. J. Physiol. 270:R1163-R1177, 1996.
16. Casley-Smith JR. The functioning and interrelationships of blood capillary and lymphatics. Experientia. 32:1-12, 1976.

17. Chang, R., Foster AH, Gann DS. Endothelial secretion of PAI following cardio-pulmonary bypass. Clin. Res. 42:883, 1994.
18. Chaudry IH, Clemens MG, Baue AE. Alterations in cell function with ischemia and shock and their correction. Arch. Surg. 116:1309-1317, 1981.
19. Chaudry IH. Cellular mechanisms in shock and ischemia and their correction. Am. J. Physiol. 245:R117-R134, 1992.
20. Chomczynski, P. Sacchii N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem., 162: 156-159, 1987.
21. Darlington DN, Keil LC, Dallman MF. Potentiation of hormonal responses to hemorrhge and fasting, but not hypoglycemia in conscious adrenalectomized rats. Endocrinology 125:1398-1398, 1989.
21. Darlington DN, Barraclough CA, Gann DS. Hypotensive hemorrhage elevates CRH mRNA but not vasopressin mRNA in the rat hypothalamus. Endocrinol. 130:1281-1288, 1992.
22. Darlington DN, Mains RE, Eipper BA. Location of neurons that express regulated endocrine-specific protein-18 in the rat diencephalon. Neuroscience 71:477-488, 1996.
23. Darlington, DN, Schiller MR, Mains RE, Eipper BA. Expression of RESP18 in peptidergic and catecholaminergic neurons. *Journal of Histochemistry and Cytochemistry in press.*
24. Darlington, DN, Schiller MR, Mains RE, Eipper BA. The expression of RESP18 in peptidergic cells of rat peripheral endocrine tissues and in blood. *Journal of Endocrinology in press.*
25. Declerck PJ, De Mol M, Vaughan DE, Collen D. Identification of a conformationally distinct form of plasminogen activator inhibitor-1 acting as a non-inhibitory substrate for tissue -type plasminogen activator. J. Biol. Chem. 267:11693-11696, 1992.
26. Dosne AM, Dubor F, Lutcher F, Parant M, Chedid L. Tumor necrosis factor (TNF) stimulates plasminogen activator inhibitor (PAI1) production by endothelial cells and decreases blood fibrinolytic activity in the rat. Thromb. Res. Suppl. 8:115-122, 1988.
26a. Drugas GT, Paidas CN, Yahantda AM, Ferguson D, Clemens MG. Conjugated desferrioxamine attenuates hepatic microvascular injury following ischemia/reperfusion. Circ Shock 34:278-283, 1991.
27. duBell WH, Lewartowski B, Spurgeon HA, Silverman HS and Lakatta EG. Repletion of sarcoplasmic reticulum Ca after ryanodine in rat ventricular myocytes. Am. J. Phyiol. 265:H604-H615,1993.
28. duBell WH, Wright PA, Lederer WJ, Rogers TB. Effect of the immunosupressant FK506 on excitation-contraction coupling and outward K+ currents in rat ventricular myocytes. Journal of Physiology. 501.3:509-516,1997.
29. Eastridge, BJ, Darlington DN, Evans JA, Gann DS. A circulating shock protein depolarizes cells in hemorrhage and sepsis. Annals of Surgery, 219:298-305, 1994.
30. Eastridge BJ, Evans JA, Darlington DN, Gann DS. Ferric iron potentiates cell depolarization by a circulating shock protein. Archives of Surgery 129:245-251, 1994.

31. Emeis JJ, Hoekzema R. de Vos AF. Inhibiting interleukin-1 and tumor necrosis factor-a does not reduce induction of plasminogen activator inhibitor type-1 by endotoxin in rats in vivo. Blood 85:115-120, 1995.

32. Esmann, M. ATPase and phosphatase activity of Na^+,K^+-ATPase: Molar and specific activity, protein determination. Meth. Enzymol. 156:105-109, 1988.

33. Evans JA, DN Darlington, and DS Gann. Circulating factor(s) mediates cell depolarization in hemorrhagic shock. Ann. Surg. 213:549-557, 1991.

34. Fagin KD, Shinsako J, Dallman MF. Effects of housing and chronic cannulation on plasma ACTH and corticosterone in the rat. Am. J. Physiol. 245:E515-E520, 1983.

35. Fukumoto S, Allan EH, Zeheb R, Gelehrter TD, Martin TJ. Glucocorticoid regulation of plasminogen activator inhibitor-1 messenger ribonucleic acid and protein in normal and malignant rat osteoblasts. Endocrinology 130:797-804, 1992.

36. Gann DS, Carlson DE, Byrnes GJ, Pirkle JC, Allen-Rowlands CF. Impaired restitution of blood volume after large hemorrhage. J. Trauma. 21:598-603, 1981.

37. Gdula WJ, Darlington DN, Butler, JM, McCord, BR, Gann DS. Measurement of rat CRH mRNA expression during hemorrhage using quantitative PCR and capillary electrophoresis. Society for Neuroscience, 1995, San Diego, (abstract 546-2).

38. Gdula, WJ, Darlington DN, Flocco M. Gann DS. Application of Capillary electrophoresis to the competitive PCR analysis of rat CRH mRNA expression during adrenalectomy. Sixth Annual Frederick Conference on Capillary Electrophoresis, 1995, (abstract p. 98).

39. Gelehrter TD, Sznycer-Laszuk R, Zeheb R, Cwikel BJ. Dexamethasone inhibition of tissue plasminogen activator (tPA) activity: paradoxical induction of both tPA antigen and plasminogen activator inhibitor. Mol. Endocrinol. 1:97-101, 1987

40. Gil A, Knockaert I, Declerck PJ. Substrate behavior of plasminogen activator inhibitor-1 is not associated with a lack of insertion of the reactive site loop. Biochemistry 35:7474-7481, 1996.

41. Heaton JH, Kathju S, Gelehrter TD. Transcriptional and posttranscriptional regulation of type 1 plasminogen activator inhibitor and tissue-type plasminogen activator gene expression in HTC rat hepatoma cells by glucocorticoids and cyclic nucleotides. Mol. Endocrinol. 6:53-60, 1992.

42. Heaton JKH. Nebes VL, O'Dell LG, Morris SM Jr., Gelehrter TD. Glucocorticoid and cyclic nucleotide regulation of plasminogen activator and plasminogen activator-inhibitor gene expression in primary cultures of rat hepatocytes. Mol. Endocrinol. 3:185-192, 1989.

43. Henninger H., Hoffmann, R, Grewe K, Schulze-Specking A, Decker K. Purification and quantitative analysis of nucleic acids by anion-exchange high-performance liquid chromatography. Biol. Chem., Hoppe-Seyler 374: 625-634, 1993.

44. Hill SA, Shaughnessy SG, Joshua P, Ribau J, Austin RC, Podor TJ. Differential mechanisms targeting type 1 plasminogen activator inhibitor and vitronectin into the storage granules of a human megakaryocytic cell line. Blood 87:5061-5073, 1996.

45. van Hinsbergh VW, van den Berg EA, Fiers W, Dooijewaard G. Tumor necrosis factor induces the production of urokinase-type plasminogen activator by human endothelial cells. Blood 75:1991-1998, 1990.

46. van Hinsbergh VW, Kooistra T, van den Berg EA, Princen HM, Fiers W, Emeis JJ.

Tumor necrosis factor increases the production of plasminogen activator inhibitor in human endothelial cells in vitro and in rats in vivo. Blood 72:1467-1473, 1988.

47. Hirota K. On the restitution of the blood fluid after hemorrhage. J. Biochem. 9:87-102, 1928.

48. Jones RO, Carlson DE, Gann DS. A circulating shock protein that depolarizes cells in vitro depresses myocardial contractility and rate in isolated rat hearts. J.Trauma 37:752-758, 1994.

49. Kirby MS, Sagara Y, Gaa S, Inesi G, Lederer WJ and Rogers TB. Thapsigargin inhibits contraction and Ca^{2+} transient in cardiac cells by specific inhibition of the sarcopolasmic reticulum Ca^{2+} pump. J. Biol. Chem. 267:12545-12551, 1992.

50. Komminoth P, and Long AA. In-situ polymerase chain reaction. Virchows Archiv B Cell Pathol 1993; 64: 67-73.

51. Konkle B, Schuster SJ, Kelly MD, Harjes K, Hassett, DE, Bohrer, M, Tavassoli M. Plasminogen activator inhibitor-1 messenger RNA expression is induced in rat hepatocytes in vivo by dexamethasone. Blood, 79: 2636-2642, 1992.

52. Krishnamurti C, Alving BM. Plasminogen activator inhibitor type 1: Biochemistry and evidence for modulation of fibrinolysis in vivo. Semin. Thromb. Hemost. 18: 67-80, 1992.

53. McKinney M, Robbins M. Chronic atropine administration up-regulates rat cortical muscarinic m1 receptor mRNA molecules: assessment with the RT/PCR. Mol. Brain Res., 12: 37-45, 1992.

54. Lindahl TL, Ohlsson P-I, Wiman B. The mechanism of the reaction between human plasminogen-activator inhibitor 1 and tissue plasminogen activator. Biochem. J. 265:109-113, 1990.

55. Medcalf RL, van den Berg E, Schleuning WD. Glucocorticoid-modulated gene expression of tissue- and urinary-type plasminogen activator and plasminogen activator inhibitor 1 and 2. J. Cell Biol. 106:971-978, 1988.

56. Medcalf RL, Druithof EK, Schleuning WD. Plasminogen activator inhibitor 1 and 2 are tumor necrosis factor/cachectin-responsive genes. J. Exper. Med. 168:751-759, 1988.

57. Medina R, Socher SH, Han JH, Friedman PA. Interleukin-1, endotoxin or tumor necrosis factor/cachectin enhance the level of plasminogen activator inhibitor messenger RNA in bovine aortic endothelial cells. Thrombosis Research. 54:41-52, 1989.

58. Mottonen J, Strand A, Symersky J, Sweet RM, Danley DE, Deoghegan KF, Gerard RD, Goldsmith EJ. Structural basis of latency in plasminogen activator inhibitor-1. Nature 355:270-273, 1992.

59. Munch M, Heegaard CW, Andreasen PA. Interconversions between active, inert and substrate forms of denatured/refolded type-1 plasminogen activator inhibitor. Biochim. Biophys. Acta 1202:29-37, 1993.

60. Niedbala MJ, Stein-Picarella M. Tumor necrosis factor regulation of endothelial cell extracellular proteolysis: the role of urokinase plasminogen activator. Biol. Chem. 373:555-566, 1992.

61. Nielsen LS, Andreasen PA, Grondahl-Hansen J, Skriver L, Dano K. Plasminogen activators catalyse conversion of inhibitor from fibrosarcoma cells to an inactive

form with a lower apparent molecular mass. FEBS Lett. 196:269-273, 1986.

62. Nuovo GJ, Lindonnichi K, MacConnell P, and Lane B. Intracellular localization of polymerase chain reaction (PCR)-amplified hepatitis C cDNA. Am J Surg Pathol 1993; 17: 683-90.

63. Okusawa S, Gelfand FA, Ikejima T, Connolly RJ, Dinarello CA. Interleukin-1 induces a shock-like state in rabbits. J. Clin. Invest. 81:1162-1172, 1988.

64. Philippe J, Offner F, Declerck PJ, Leroux-Roels G, Vogelaers D, Baele G, Collen D. Fibrinolysis and coagulation in patients with infectious disease and sepsis. Thromb. Haemost. 65:291-295, 1991.

65. Pirkle JC, Gann DS. Restitution of blood volume after hemorrhage: role of the adrenal cortex. Am. J. Physiol. 230:1683-1687, 1976.

66. Pirkle JC, Gann DS. Expansion of interstitial fluid is required for full restitution of blood volume after hemorrhage. J. Trauma. 16:937-947, 1976.

67. Podor TJ, Hirsh J, Gelehrter TD, Zeheb R, Torry D, Guigoz Y, Sierra F, Gauldie J. Type 1 plasminogen activator inhibitor is not an acute phase reactant in rats. Lack of IL6- and hepatocyte-dependent synthesis. J. Immunology 150:225-235, 1993.

68. Pralong G, Calandra T, Glauser MP, Schellekens J, Verhoef J, Bachmann F, Kruithof EK. Plasminogen activator inhibitor 1: a new prognostic marker in septic shock. Thromb. Haemost. 61:459-462, 1989.

69. Preissner KT. Structure and biological role of vitronectin. Annu. Rev. Cell Biol. 7:275-310, 1991.

70. Preissner KT, Wassmuth R, Muller-Berghaus G. Physico-chemical characterization of human S-protein and its function in the blood coagulation system. Biochem J. 231:349-355, 1985.

71. Saksela O, Rifkin DB. Cell-associated plasminogen activation: Regulation and physiological functions. Ann Rev Cell Biol. 4:93-126, 1988.

72. Sawdey MS, Loskutoff DJ. Regulation of murine type 1 plasminogen activator inhibitor gene expression in vivo. Tissue specificity and induction by lipopolysaccharide, tumor necrosis factor-alpha, and transforming growth factor-beta. J. Clin. Invest. 88:1346-1353, 1991.

73. Seeber C, Hiller E. Holler E, Kolb HJ. Increased levels of tissue plasminogen activator (tPA) and tissue plasminogen activator inhibitor (PAI) correlate with tumor necrosis factor alpha (TNF a)-release in patients suffering from microangiopathy following allogeneic bone marrow transplantation (BMT). Thrombosis Research 66:373-383, 1992.

74. Shimaya K, Sumi H, Maruyama M, Mihara H. A rapid and strong release of plasminogen activator induced by experimental anaphylaxis in rabbits. Enzyme 46:204-212, 1992.

75. Shires GT, Cunningham JN, Baker CRF. Alterations in cellular membrane function during hemorrhagic shock in primates. Ann. Surg. 176:288-295, 1972.

76. Shires GT III, Peitzman AB, Illner H, Shires GT. Changes in red blood cell transmembrane potential, electrolytes and energy content in septic shock. J. Trauma. 23:769-774, 1983.

77. Siebeck M, Spannagl M, Hoffman H, Schramm W, Fritz H. Time dependent release of tissue-type plasminogen activator and plasminogen activator inhibitor into the

circulation of pigs during shock. Blood Coagul. Fibrinol. 2:459-464, 1991.

78. Slivka SR, Loskutoff DJ. Regulation of type I plasminogen activator inhibitor synthesis by protein kinase C and cAMP in bovine aortic endothelial cells. Biochimica Biophysica ACTA 1094:317-322, 1991.

79. Starling, EH. On the absorption of fluids from the connective tissue spaces. J. Physiol. (Lond.) 19:312-326, 1896.

80. Tracey KJ, Fong Y, Hesse DB, Manoque KR, Lee AT, Kuo GC, Lowry SF, Cerami A. Anti-cachectin/TNF monoclonal antibodies prevent septic shock during lethal bacteraemia. Nature. 330:662-664, 1987.

81. Trump BF, Berezesky IK. Calcium-mediated cell injury and cell death. FASEB Journal. 9:219-28, 1995

82. Wakabayashi G, Gelfand FA, Burke JF, Thompson RC, Dinarello CA. A specific receptor antagonist for interleukin-1 prevents E. coli induced shock in rabbits. FASEB J. 5:338-343, 1991.

83. Ware J, Ljungqvist O, Norberg KA, Nylander G. Osmolary changes in haemorrhage: the effects of an altered nutritional status. Acta Chir. Scand. 148:641-646, 1982.

84. Whawell, S.A., D.M. Scott-Coombes, M.N. Vipond, S.J. Tebbutt, J.N. Thompson. Tumor necrosis factor-mediated release of plasminogen activator inhibitor 1 by human peritoneal mesothelial cells. Br. J. Surg., 81:214-216, 1994.

85. Whawell, S.A. and J.N. Thompson. Cytokine-induced release of plasminogen activator inhibitor-1 by human mesothelial cells. Eur. J. Surg. 161:315-317, 1995.

86. Whipple GH. Protein production and exchange in the body including hemoglobin, plasma protein and cell protein. Am. J. Med. Sci. 196:609-621, 1938.

87. Wun TC, Palmier MO, Siegel NR, Smith CE. Affinity purification of active plasminogen activator inhibiotr-1 (PAI-1) using immobilized anhydrourokinase. J. biol. Chem. 264:7862-7868,1989.

88. Yamashita M, Darlington DN, Imafuku S, Gann DS. Plasminogen activator inhibitor-1 mRNA is induced by hemorrhage in endothelial and mesothelial cells of the rat liver. Thromb. Haemost., 74: 933-937, 1995.

89. Yamashita M, Darlington DN, Weeks EJ, Jones RO, Gann DS. Plasminogen activator inhibitor-1 rises after hemorrhage in rats. Am. J. Physiol., 268: E1065-E1069, 1995.

90. Zoellner H, Wojta J, Gallicchio M, McGrath K, Hamilton JA, Cytokine regulation of the synthesis of plasminogen activator inhibitor-2 by human vascular endothelial cells. Comparison with plasminogen activator inhibitor-1 synthesis. Thromb. Haemost. 69:135-140, 1993.

Bibliography:

Eastridge, B.J, D.N. Darlington, J.A. Evans and D.S. Gann. A circulating shock protein depolarizes cells in hemorrhage and sepsis. Annals of Surgery, 219:298-305, 1994.

Eastridge B.J.,- J.A. Evans, D.N. Darlington and D.S. Gann Ferric iron potentiates cell depolarization by a circulating shock protein. Archives of Surgery 129:245-251, 1994.

Jones, R.O., D.E. Carlson, D.S. Gann. A circulating shock protein that depolarizes cells in vitro depresses myocardial contractility and rate in isolated rat hearts. J. Trauma 37:752-758, 1994.

Borchelt, B. .D. , P.A. Wright, J. A. Evans and D.S . Gann. Cell swelling and depolarization in hemorrhagic shock. J. Trauma 39:187-194, 1995.

Darlington, D.N., R.O. Jones, T.A. Magnuson and D.S. Gann. The role of Intestinal fluid in the Restitution of blood volume and plasma protein after hemorrhage in awake rats. *American Journal Physiol.* 268(Regulatory, Integrative and Comparative Physiology 37): R715-R722, 1995.

Yamashita, M., D.N. Darlington, E.J. Weeks, R.O. Jones and D.S. Gann. Plasminogen activator inhibi tor type 1 message rises after hemorrhage in the rat. *American Journal of Physiology*, 268 (Endocrinology and Metabolism, 31):E1065-E1069,1995.

Darlington, D.N., R.O. Jones, Marzella and D.S. Gann. Changes in regional vascular resistance and blood volume after hemorrhage in fed and fasted awake rats. *Journal Applied Physiology* 78(6):2025-2032,1995

Yamashita, M., D.N. Darlington, S. Imafuku and D.S. Gann. Hemorrhage induces plasminogen activator inhibitor-1 mRNA in endothelial and mesothelial cells of rat liver. *Thrombosis and Hemostasis* 74:933-937,1995.

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